REMARKS

Claims 36, 39, 40, 43 and 44 were previously pending in this application. By this amendment, Applicant is canceling claim 43 without prejudice or disclaimer. Claims 36 and 40 have been amended. Support for the amendments to the claims can be found in the specification as filed at page 10, lines 24-26 (kinase-dead MLK2 inhibits neuronal cell death); page 21, lines 8-15 (inhibiting MLK kinase activity for treatment); and Example 3, particularly page 31, lines 11-18, page 32, lines 9-16, page 33, line 20 - page 34, line 12 (kinase-dead MLK2 preparation and effects in inhibiting neuronal cell death). As a result claims 36, 39, 40, and 44 are pending for examination with claims 36 and 40 being independent claims. No new matter has been added.

Applicant hereby requests a two month extension of time to and including September 30, 2004, for responding to the Office Action mailed April 30, 2004.

Rejection Under 35 U.S.C. §112

The Examiner has rejected claims 36, 39, 40, 43, and 44 under 35 U.S.C. §112, first paragraph as containing subject matter not adequately described in the specification so as to enable one skilled in the art to practice the invention.

Claims 36 and 40 have been amended to include specific compounds that inhibit MLK activity. The compounds now claimed mimic the effect of kinase-dead MLK2, which has a mutation in its ATP binding site. The specification, in describing kinase-dead MLK2, provides an implicit description that compounds that bind to the ATP binding site of MLK, are effective to inhibit MLK activity. Applicant also provides herewith a copy of *J. Biol. Chem.* 275:19035-19040 (2000), which demonstrates the effect of kinase-dead MLK2 as an inhibitor of MLK activity (see, page 19036, left column ("Subcloning and Mutagenesis" section, which indicates location of the mutation in the ATP binding site of the MLK2 kinase domain); page 19039, left column; and Fig. 4).

The identification and use of compounds that inhibit ATP binding to MLK would not require undue experimentation on the part of the ordinarily skilled artisan in view of the guidance provided in the specification of the inhibitory effect of kinase-dead MLK2 on MLK activity (e.g., Example 3) and the general knowledge in the art that compounds that bind to ATP binding sites of kinases and inhibit ATP binding to the ATP binding sites are useful as inhibitors

of the kinases (see, e.g., US 6,162,613; US 6,573,044; US 6,221,900; and references cited therein). Given that no more than routine experimentation would be required by one of ordinary skill in the art to identify and use such compounds according to the present invention, Applicant asserts that the amended claims are fully enabled.

Based on the amendments to the claims, Applicant respectfully requests that the Examiner withdraw the rejections of claims 36, 39, 40, and 44 under 35 U.S.C. §112, first paragraph.

Rejection Under 35 U.S.C. §103

The Examiner rejected claims 36, 39, 40, 43 and 44 under 35 U.S.C. §103(a) as being unpatentable over Miller et al. (US 6,060,247). Applicant has amended claims 36 and 40 and believes that the amendments obviate the rejection.

To successfully support a *prima facie* case of obviousness, the Examiner must demonstrate that the cited reference teaches all of the claimed features, that there would be motivation to modify the teaching in the reference to make the claimed invention, and that there would be a likelihood of success in making the modification. Applicant respectfully asserts that the '247 patent does not meet the requisite criteria to support the obviousness rejection. The '247 patent does not teach all of the claimed features of the invention as evidenced by the absence of any teaching regarding the claimed compounds that bind to the MLK ATP binding site and thereby inhibit MLK kinase activity, or the administration of the compounds as a treatment for Parkinson's disease or any other apoptosis-associated disorder. In addition, Applicant respectfully asserts that one of ordinary skill in the art would be not motivated to modify the general disclosure of the '247 patent to make the claimed invention.

The '247 patent describes the use of adenovirus constructs to identify compounds that either increase or decrease apoptosis, but the patent provides no teaching as to how one would select any one of the various listed adenovirus constructs or use any molecule identified as a treatment for Parkinson's disease. Applicant asserts that the general suggestions regarding apoptosis that are provided in the '247 patent cannot simply be coupled with the patent's assorted constructs to reach the claimed invention, particularly in light of the failure of the '247 patent to suggest any relationship between MLK activity and Parkinson's disease. Thus, absent a link between MLK, MLK inhibition by compounds that bind to the MLK ATP binding site and

thereby inhibit MLK kinase activity, and/or Parkinson's disease, the teaching in the '247 patent is insufficient to allow one of ordinary skill to make the claimed invention.

Because the '247 patent does not provide each and every claimed element of the invention and also fails to provide motivation for one of ordinary skill in the art to select compounds that bind to the MLK ATP binding site and thereby inhibit MLK kinase activity as a treatment for Parkinson's disease, Applicant submits that the '247 patent does not render obvious the claims as amended.

Based on the arguments presented and the amendments to the claims, Applicant respectfully requests that the Examiner withdraw the rejection of claims 36, 39, 40, and 44 under 35 U.S.C. §103(a).

CONCLUSION

In view of the foregoing amendments and remarks, this application should now be in condition for allowance. A notice to this effect is respectfully requested. If the Examiner believes, after this amendment, that the application is not in condition for allowance, the Examiner is requested to call the Applicant's attorney at the telephone number listed below.

If this response is not considered timely filed and if a request for an extension of time is otherwise absent, Applicant hereby requests any necessary extension of time. If there is a fee occasioned by this response, including an extension fee that is not covered by an enclosed check, please charge any deficiency to Deposit Account No. 23/2825.

Respectfully submitted,
Ya Fang Liu, Applicant

By: 

John R. Van Amsterdam, Reg. No.40,212
Wolf, Greenfield & Sacks, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2211
Telephone: (617) 720-3500

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Activation of MLK2-mediated Signaling Cascades by Polyglutamine-expanded Huntingtin*

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Ya Fang Liu‡§, Donna Dorow¶, and John Marshall‖

From the ‡Department of Pharmaceutical Sciences, Northeastern University, Boston, Massachusetts 02115, ¶Trecowthick Research Center, Peter MacCallum Cancer Institute, Melbourne, Victoria 3000, Australia, and ‖Department of Molecular Pharmacology, Physiology and Biotechnology, Brown University, Providence, Rhode Island 02912

We previously reported that expression of polyglutamine-expanded huntingtin induces apoptosis via c-Jun amino-terminal kinase (JNK) activation in HN33 cells (Liu, Y. F. (1998) *J. Biol. Chem.* 273, 28873–28822). Extending this study, we now demonstrate a role of mixed-lineage kinase 2 (MLK2), a JNK activator, in polyglutamine-expanded huntingtin-mediated neuronal toxicity. We find that normal huntingtin interacts with MLK2, whereas the polyglutamine expansion interferes with this interaction. Similar to the expression of polyglutamine-expanded huntingtin, expression of MLK2 also induces JNK activation and apoptosis in HN33 cells. Co-expression of dominant negative MLK2 significantly attenuates neuronal apoptosis induced by the mutated huntingtin. Furthermore, over-expression of the N terminus of normal huntingtin partially rescues the neuronal toxicity induced by MLK2. Our results suggest that activation of MLK2-mediated signaling cascades may be partially involved in neuronal death induced by polyglutamine-expanded huntingtin.

Huntington's disease (HD)[1] is a neurodegenerative disorder with dominant inheritance (2). The disease is characterized by choreiform movement, mental impairment, and cognitive symptoms (3, 4). The HD gene encodes a 350-kDa protein designated as huntingtin (2), which is ubiquitously expressed with the highest levels being found in the brain, lung, and testes (5, 6). Immunocytochemistry reveals that in neurons huntingtin is a cytoplasmic protein found in cell bodies, dendrites, and also in nerve terminals, where huntingtin is associated with synaptic vesicles and microtubule complexes (6, 7). The defect in the HD gene causes an expansion of a polyglutamine stretch near the N terminus of huntingtin, and the length of the polyglutamine repeat is correlated with the age of onset and the severity of the disease (8). To date, the normal function of huntingtin remains to be determined, and the molecular mechanism underlying neuronal death in HD is poorly understood.

In previous studies, we found that expression of polyglutamine-expanded huntingtin caused neuronal apoptosis via activation of JNKs in HN33 cells, a hippocampal neuron-derived cell line (1). The aim of the present study was to investigate the molecular mechanism by which polyglutamine-expanded huntingtin activates JNKs and induces neuronal apoptosis. Huntingtin contains multiple proline-rich motifs that may bind to both SH3 and WW domain-containing proteins (9). Interestingly, the N-terminal proline-rich region, which is about 40 amino acids long, is adjacent to the polyglutamine stretch. This proline-rich region has been shown to bind to both SH3 and WW domain-containing proteins (10–12). MLK2 is a member of the mixed-lineage kinase family whose kinase domain shows structural features of both tyrosine-specific and serine/threonine-specific protein kinases (13). MLK2 possesses an SH3 domain that is homologous to the SH3 domains of Grb2 (13). MLK2 is predominantly expressed in the brain (13), and it has been reported that MLK2 can directly bind and mediate activation of MKK7 and SEK1, which in turn induces JNK activation (14–19). At moderate expression levels, MLK2 appears to selectively activate JNKs and has little effect on other mitogen-activated protein kinases (14–16). Thus, MLK2 is a potential candidate for the involvement in JNK activation and neuronal toxicity induced by polyglutamine-expanded huntingtin. The current study was undertaken to investigate the role of MLK2 in mutated huntingtin-mediated neuronal toxicity. Our results suggest that huntingtin binds to the SH3 domain of MLK2 and the polyglutamine expansion interferes with its binding to the kinase. Activation of MLK2-mediated signal transduction pathways may be involved in initiating neuronal death in HD.

MATERIALS AND METHODS

Cell Culture and Transient Transfection—HN33 cells, an immortalized rat hippocampal neuronal cell line (1), and 293T cells, human embryonic kidney cells expressing SV40 large T antigen, were maintained in Dulbecco's modified Eagle's medium/F12 medium supplemented with 10% fetal bovine serum on 10-cm plates. HN33 or 293T cells at 50 to 60% confluence were washed once with serum-free medium prior to transfection. Transfection was performed using Lipofectin (Life Technologies, Inc.) according to manufacturer instructions. 10–50 μg of plasmid with 10–20 μl of Lipofectin/10-cm plate was used in transfection experiments.

Western Blotting and Immunoprecipitation—48–72 h after transfection, 293T cells were harvested and lysed in 1% Nonidet P-40 lysis buffer, and co-immunoprecipitation experiments were conducted as described previously using an anti-huntingtin's N terminus antibody 437 (10) or anti-c-Myc-tagged antibody 9E10 (Santa Cruz). Human brain tissues were obtained from Dr. J.-P. Vonsattel or Human Brain Bank at McLean Hospital, Boston, MA with institutional review board approval. Post-mortem time was between 10–12 h. The diagnosis of HD was confirmed with neuropathological and genetic phenotype analysis. Human cortex tissues from normal subjects or HD patients were ho-

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This work is dedicated to Dr. R.-Y. Zhong.

§ To whom correspondence should be addressed: Dept. of Pharmaceutical Sciences, Northeastern University, 312 Mugar Hall, 360 Huntington Ave., Boston, MA 02115. Tel.: 617-373-3904; Fax: 617-373-8886; E-mail: yafliu@lynx.neu.edu.

[1] The abbreviations used are: HD, Huntington's disease; MLK2, mixed lineage kinase 2; JNK(s), c-Jun amino-terminal kinase(s); MKK7, mitogen-activated protein kinase 7; SEK1, stress-signaling kinase 1; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; TUNEL, Tdt-mediated dUTP-biotin nick end labeling; IP, immunoprecipitation.

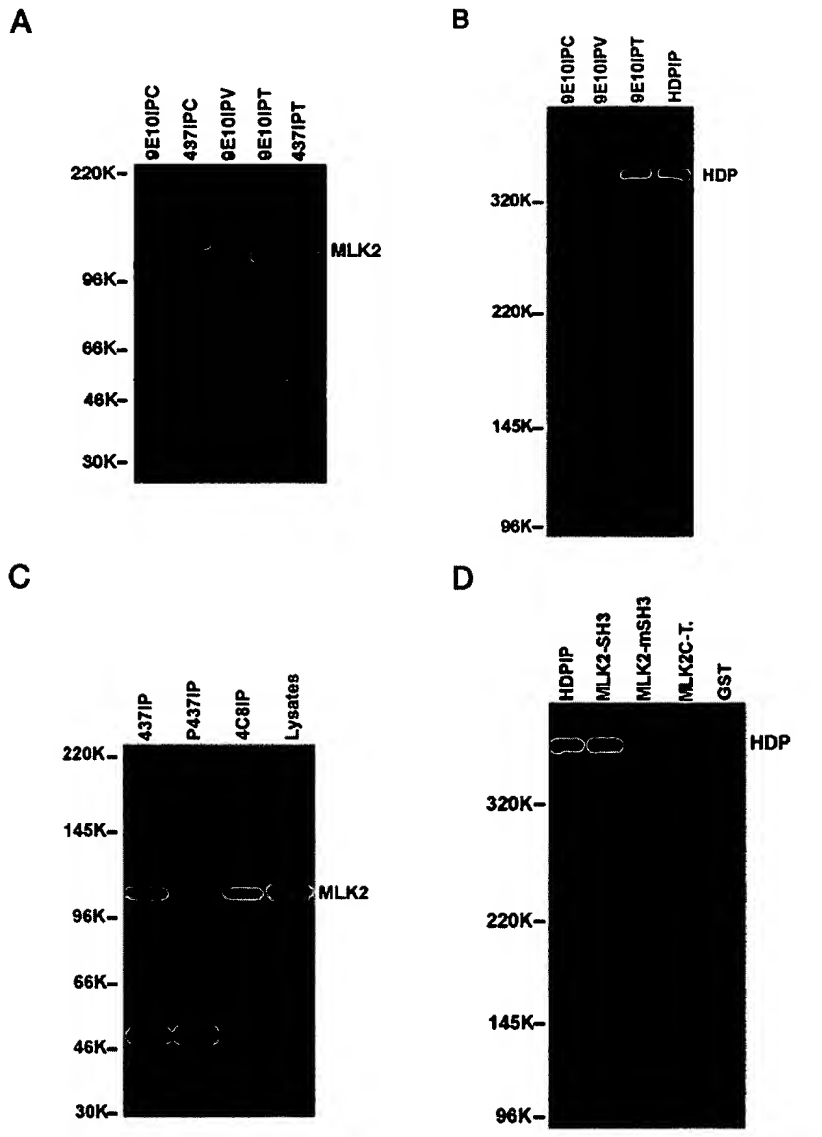

FIG. 1. **The association of normal huntingtin with MLK2.** All data presented are from a typical experiment that has been repeated twice with similar results. *A*, detection of MLK2 in huntingtin immunoprecipitates was as follows: 293T cell lysates of wild-type (*437IPC* and *9E10IPC*) or transfected with pRK5 vector alone (*9E10IPV*) or c-Myc-tagged MLK2 (*437IPT* and *9E10IPT*) were incubated with 9E10 or 437, and the blot was analyzed by 9E10. *B*, detection of huntingtin in MLK2 immunoprecipitates was as follows: 293T cell lysates of wild-type (*9E10IPC*) or transfected with vector alone (*9E10IPV*) or c-Myc-tagged MLK2 (*9E10IPT*) were incubated with 9E10, and the blot was analyzed by 4C8 (3). 437 immunoprecipitation (*HDPIP*) was a positive control. *HDP*, huntingtin. *C*, detection of endogenously expressed MLK2 in huntingtin immunoprecipitates was as follows: HN33 cell lysates were incubated with 437 or 4C8, and the blot was analyzed by an anti-MLK2 antibody characterized previously (36). *437IP*, IP with 437; *P437IP*, IP with peptide-antigen preabsorbed 437; *4C8IP*, IP with 4C8. *D*, the SH3 domain of MLK2 mediated its interaction with huntingtin as follows: GST alone or GST fusion proteins of MLK2-SH3 domain (*MLK2-SH3*), MLK2-C terminus (*MLK2C-T.*), or MLK2-deficient SH3 domain (*MLK2-mSH3*) were incubated with 293T cell lysates, and the blot was analyzed by 437.

mogenized in detergent-free lysis buffer, and Nonidet P-40 was added to a final concentration of 1%. The mixture was then incubated at 4 °C with constant shaking for 1–2 h, and insoluble fractions were removed by centrifugation.

Purification of GST Fusion Proteins and in Vitro Binding Assay—Huntingtin N-terminal GST fusion protein constructs were generated by subcloning of a cDNA fragment encoding the first three exons of the HD gene containing 16 or 56 CAG repeats into pGEX2T. Construct for MLK2 C terminus (amino acids 407–953) GST fusion proteins was a generous gift of Dr. Alan Hall (University College London, London, UK) (14). The MLK2 SH3 domain cDNA fragment was amplified by reverse transcription-PCR and inserted into pGEX4T1. To generate MLK2 SH3-deficient GST fusion proteins, substitution of the first tryptophan at position 58 of the highly conserved tryptophan doublet of the SH3 domain to lysine was achieved by overlapping extension using PCR with mutated oligonucleotides. Such a substitution eliminates the ability of SH3 domains to bind to proline-rich ligands (10). Expression and purification of different MLK2 or huntingtin GST fusion proteins was performed as described previously, and ~0.1 μg of GST fusion protein was used for *in vitro* binding studies.

Subcloning and Mutagenesis—The full-length c-Myc-tagged MLK2 was a gift from Dr. Alan Hall (14). A kinase-dead version of MLK2 was generated by introduction of an Ala-Gly point mutation at position 651 (codon AAG to GAG) by overlapping PCR extension with mutated oligonucleotides, to result in an amino acid substitution of Lys to Glu at the ATP binding loop of the kinase domain. To generate an expression vector for huntingtin's N terminus with 16 CAG repeats, a cDNA fragment was excised from pFL16HD with *Not*I and *Sph*I and subcloned into pcDNA 1.1 (Invitrogen).

JNK and TUNEL Assays—16 h after transfection, HN33 cells were lysed with 1% Triton X-100 lysis buffer (1). JNK was assayed as described previously (1). For TUNEL assay, HN33 cells were plated on a slide culture chamber. After transfection, cells were fixed at the time indicated in the figures, and TUNEL staining was performed as described previously (1). Most apoptotic HN33 cells were detached from the slides, and TUNEL stain was performed on remaining attached cells. TUNEL stain-negative cells (living cells) were counted in the 20× power field in four different locations on the slides and ~600–800 cells were counted in the control (1).

RESULTS

293T cells, which are rich in huntingtin (10), were utilized to study the interaction of huntingtin with MLK2. c-Myc-tagged MLK2 was transiently expressed in 293T cells, and cell lysates were immunoprecipitated with 437, an anti-huntingtin N-terminal antibody (10), and the resulting blot was probed with 9E10, an anti-c-Myc-tagged monoclonal antibody. MLK2 was detected in 437 and 9E10 immunoprecipitates of lysates transfected with c-Myc-tagged MLK2 but not in the negative controls, 9E10 or 437 immunoprecipitates of wild-type or vector-transfected 293T cell lysates (Fig. 1*A*). Conversely, we also found that huntingtin protein was present in 9E10 immunoprecipitates of c-Myc-tagged MLK2-transfected 293T cell lysates but not wild-type or vector-transfected lysates (Fig. 1*B*).

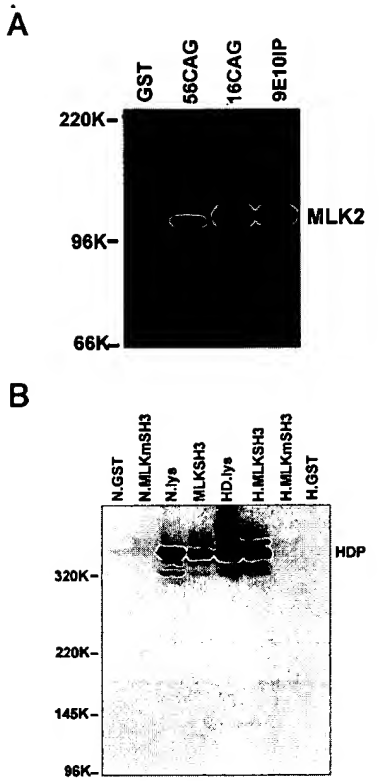

FIG. 2. **The polyglutamine expansion interferes with huntingtin to bind to the SH3 domain of MLK2.** All data presented are from a typical experiment that has been repeated at least three times with similar results. *A*, the polyglutamine expansion inhibits huntingtin's N terminus to bind to MLK2; 293T cell lysates transfected with c-Myc-tagged MLK2 were incubated with GST alone or huntingtin's N terminus GST fusion proteins containing 16 (*16CAG*) or 56 (*56CAG*) polyglutamine repeats, and the blot was analyzed by 9E10 (*9E10IP*). *B*, the ability of the mutated huntingtin to bind to the SH3 domain of MLK2 is impaired. Human cortex lysates from a normal subject and an HD patient were incubated with GST alone or GST fusion proteins of MLK2-SH3 domain (*MLKSH3*) or MLK2-deficient SH3 domain (*MLKmSH3*), and the blot was analyzed by 437. *N. lys* or *N.*, normal human cortex lysates; *HD. lys* or *H.*, HD patient's cortex lysates.

These data show that MLK2 is associated with huntingtin in intact cells.

Next, we examined the interaction of MLK2 with huntingtin in a hippocampal neuronal cell line, HN33 cells in which we found that expression of polyglutamine-expanded huntingtin induced apoptosis (1). MLK2 is richly expressed in HN33 cells, and the amount of MLK2 proteins in the cell is similar to that of 293T cells over-expressing c-Myc-tagged MLK2 (Fig. 1*C*). HN33 cell lysates were immunoprecipitated with 437 or 4C8, a well characterized anti-huntingtin monoclonal antibody (5), and the resulting blot was probed with a specific anti-MLK2 antibody that has been characterized previously (20). As shown in Fig. 2*B*, both 437 and 4C8 were able to precipitate MLK2 from HN33 cell lysates, whereas the peptide-antigen pre-absorbed 437 failed to do so. These data provide further evidence that normal huntingtin is associated with MLK2 in neuronal cells.

To determine whether the SH3 domain of MLK2 mediates its interaction with huntingtin, we investigated the binding of huntingtin to a GST MLK2-SH3 domain fusion protein, whereas a GST MLK2 C terminus fusion protein, which lacks the SH3 domain, served as a negative control. As shown in Fig. 1*D*, huntingtin binds to the MLK2 SH3 domain but not GST alone or the MLK2 C terminus. To verify whether the SH3 domain of MLK2 mediates its interaction with huntingtin, we generated a MLK2-SH3 domain-deficient GST fusion protein by substitution of the first tryptophan of the highly conserved tryptophan doublet of the MLK2 SH3 domain to lysine. Mutation of the tryptophan doublet of the SH3 domain is known to eliminate its ability to bind to proline-rich ligands (10). Huntingtin failed to bind to MLK2-SH3 domain-deficient GST fusion proteins (Fig. 1*D*). These data show that the SH3 domain of MLK2 mediates its interaction with normal huntingtin.

We next determined whether expansion of the polyglutamine repeat in huntingtin would alter its interaction with MLK2. The N-terminal proline-rich region adjacent to the polyglutamine repeat has been reported to bind to SH3 domain-containing proteins (10–11). Thus, we examined the binding of MLK2 to huntingtin's N terminus containing either a normal or expanded polyglutamine stretch. GST fusion proteins of huntingtin's N terminus containing 16 or 56 polyglutamine repeats were generated and purified. These GST fusion proteins were utilized as a template to examine whether huntingtin's N terminus is responsible for its interaction with MLK2 and how expansion of the polyglutamine repeat affects this interaction. As shown in Fig. 2*A*, MLK2 binds to huntingtin's N terminus containing 16 polyglutamine repeats. Because the N-terminal proline region is the only potential SH3 domain binding site in this small N-terminal segment of huntingtin and others have shown that this region mediates huntingtin binding to SH3 domain (11), these data suggest that the N-terminal proline region is involved in huntingtin interaction with MLK2. The amount of c-Myc-tagged MLK2 bound to huntingtin's N terminus with 56 polyglutamine repeats was significantly reduced, about 70% less than that associated with the N terminus of normal huntingtin (Fig. 2*A*). This data indicates that expansion of the polyglutamine repeat may inhibit the ability of huntingtin's N terminus to interact with the SH3 domain of MLK2.

Next, we examined the interaction of MLK2 with huntingtin in the human brain. Because our MLK2 antibody cannot be used for immunoprecipitation, a MLK2 SH3 domain GST fusion protein was used to test the ability of normal and polyglutamine-expanded huntingtin proteins from human brain tissues to bind to MLK2. Lysates of human brain cortex tissues from a normal subject and a mid-age onset HD patient were prepared. Wild-type or mutated MLK2 SH3 domain GST fusion protein was incubated with human brain lysates, and the resulting blot was probed with 437. As shown in Fig. 2*B*, normal huntingtin protein from normal or HD human cortex tissues bound to the wild-type MLK2 SH3 GST fusion protein but not to GST alone or to the MLK2 SH3 domain-deficient GST fusion protein. In contrast, polyglutamine-expanded huntingtin protein from the HD patient brain only weakly bound to the SH3 domain of MLK2 (Fig. 2*B*). These data further support our findings that normal huntingtin binds to the SH3 domain of MLK2 and that the polyglutamine expansion interferes with its ability to interact with the SH3 domain of the kinase.

MLK2 is known to induce JNK activation in Cos-1 cells (14). Therefore, we tested whether expression of MLK2 activates JNKs in HN33 cells. The MLK2 expression vector (pRK5) or vector alone was transiently transfected into HN33 cells. JNKs were precipitated using a GST-c-Jun protein, and an *in vitro* JNK assay was performed. An equal amount of JNK proteins were precipitated in each JNK assay (data not shown). As observed in other neuronal cells (21), a basal level of JNK activity was found in HN33 cells (Fig. 3*A*). MLK2 induced constitutive activation of JNKs in HN33 cells. As shown in Fig. 3*A*, the level of the JNK activity was increased by 8-fold (Fig. 3, *A* and *B*). Because MLK2 mediates JNK activation via phosphorylation and activation of both MKK7 and SEK1 (14–16), we determined whether co-expression of dominant negative

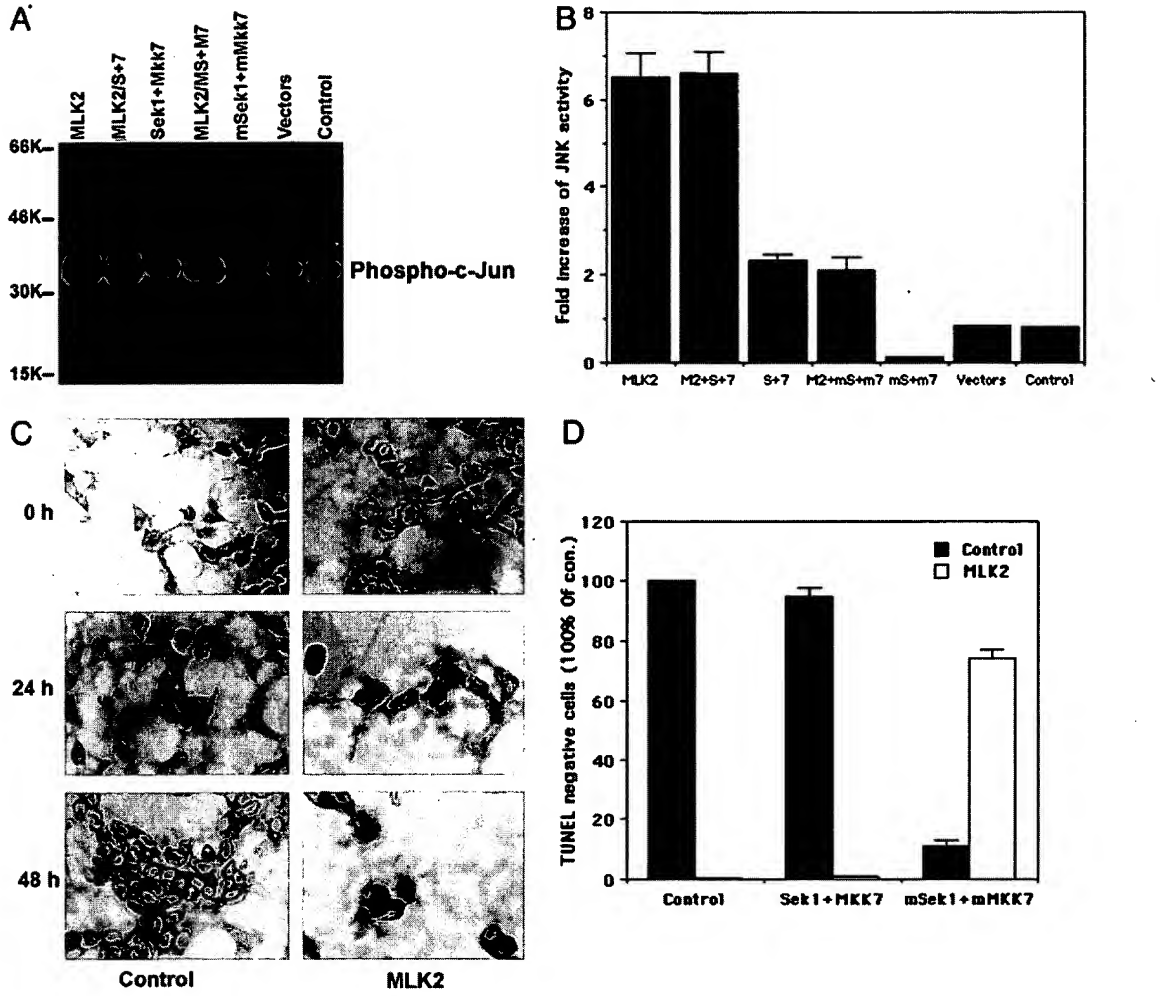

FIG. 3. **Expression of MLK2 in HN33 cells induces JNK activation and neuronal apoptosis.** *A*, expression of MLK2-mediated JNK activation in HN33 cells; HN33 cells were transiently transfected with different plasmids as indicated in the figure. 16 h after transfection, HN33 cells were lysed, and JNK activity was measured as described previously (24). *S*, Sek1; *7*, MKK7, *MS*, dominant negative Sek1 (24); *M7*, dominant negative MKK7; *vectors*, pRK5 and pEBG. *B*, the -fold of the JNK activity induced by MLK2. Increase of JNK activity was determined by analyzing the blots with a densitometer. The values depicted represent the -fold stimulation of JNK activity of HN33 cells transfected with different plasmids as indicated in the figure over the activity of HN33 cells transfected with vector alone. Data are the average of three independent experiments. *C*, expression of MLK2 in HN33 cells induced apoptotic cell death. HN33 cells were transfected with MLK2 expression vector. Following transfection, cells were fixed at the times indicated in the figure, and TUNEL staining was performed as described under "Materials and Methods." Most apoptotic HN33 cells were detached from slides, and TUNEL staining was performed on the remaining cells. Cells showing the retraction of neurites and positive stain in the nucleus were recognized as apoptotic. TUNEL-negative cells (living cells) were counted, and the number of TUNEL-negative cells in the control (transfected with pRK5 + pEBG + pcDNA1) was designated as 100%. Data are the average of three independent experiments. *D*, co-expression of dominant negative MKK7 and SEK1 significantly inhibited MLK2-mediated apoptosis in HN33 cells. HN33 cells were co-transfected with MLK2 expression vector and wild-type or dominant negative SEK1 or MKK7. TUNEL staining was conducted, and TUNEL-negative cells were counted.

MKK7 and SEK1 could block MLK2-mediated JNK activation. As shown in Fig. 3*A*, MLK2-mediated JNK activation was significantly attenuated by co-expression of dominant negative MKK7 and SEK1 but not by co-expression of wild-type MKK7 and SEK1, which had little effect on the JNK activity induced by MLK2 (Fig. 3, *A* and *B*). Co-expression of wild-type MKK7 and SEK1 did not significantly alter basal JNK activity in HN33 cells (Fig. 3, *A* and *B*), whereas co-expression of dominant negative MKK7 and SEK1 inhibited basal JNK activity, which is toxic to HN33 cells (see below Fig. 3*D*).

We have reported that expression of polyglutamine-expanded huntingtin induces neuronal apoptosis by activation of JNKs in HN33 cells (1). Because MLK2 also activates JNKs in HN33 cells, we examined whether expression of MLK2 induced neuronal toxicity in HN33 cells. Vector alone or c-Myc-tagged MLK2 was transiently expressed in HN33 cells and, 24 and 48 h post-transfection, cells were fixed and Tdt-mediated dUTP-biotin nick end labeling (TUNEL) staining, which detects the late stage of apoptosis, was conducted. TUNEL-negative cells (living cells) were counted. Expression of MLK2 induced rapid apoptotic cell death in HN33 cells (Fig. 3*C*). HN33 cells started to undergo apoptosis at ~24 h post-transfection (Fig. 3*C*). At 48 h post-transfection, most HN33 cells were detached from the plate (apoptotic), and remaining attached cells were also apoptotic (Fig. 3*C*). Similar to expression of polyglutamine-expanded huntingtin (1), expression of MLK2 not only led to neuronal death of transfected HN33 cells but also mediated cell toxicity of non-transfected HN33 cells, suggesting that activation of MLK2 may cause the release of cellular diffusible factors that are toxic to neighboring neuronal cells (1).

Next, we investigated whether co-expression of dominant negative MKK7 and SEK1 would inhibit MLK2-mediated neuronal toxicity. Expression of wild-type or dominant negative mutant forms of MKK7 or SEK1 alone did not alter cell viability (1). Co-expression of wild-type SEK1 and MKK7 also did not

generate any cell toxicity (Fig. 3*D*), whereas co-expression of dominant negative SEK1 and MKK7 significantly attenuated neuronal toxicity induced by MLK2. As shown in Fig. 3*D*, ~75% of HN33 cells were rescued upon co-expression of the dominant negative mutant form of MKK7 and SEK1. These data indicate that JNK-mediated neuronal toxicity is induced by MLK2 in HN33 cells. Co-expression of dominant negative MKK7 and SEK1 without MLK2 caused rapid apoptosis in HN33 cells (Fig. 3*D*). Inhibition of basal JNK activity may account for this cell toxicity, because co-expression of dominant negative MKK7 and SEK1 decreased basal JNK activity in HN33 cells (Fig. 3, *A* and *B*), whereas when these two mutated kinases were co-expressed with MLK2 in HN33 cells, JNK activity was double the basal level (Fig. 3, *A* and *B*), and under this condition HN33 cells were viable (Fig. 3*D*). These results suggest that a certain basal level of JNK activity appears to be essential for the survival of HN33 cells, and either over-activation or inhibition of basal JNK activity triggers apoptosis.

To further investigate whether MLK2-mediated signaling cascades are involved in neuronal death induced by polyglutamine-expanded huntingtin, a dominant negative (kinase-dead) form of MLK2, which is known to competitively inhibit the endogenous kinase, was generated. Different full-length huntingtin expression vectors containing 16, 48, or 89 polyglutamine repeats were separately co-transfected with wild-type or the dominant negative form of MLK2 into HN33 cells. As shown in Fig. 4*A*, co-expression of dominant negative MLK2 significantly inhibited neuronal toxicity mediated by polyglutamine-expanded huntingtin in HN33 cells. At 48 h post-transfection of the huntingtin construct containing 48 or 89 CAG repeats, over 75% of HN33 cells remained viable (*i.e.* trypan blue stain-negative) when dominant negative MLK2 is co-expressed, compared with 70–80% of apoptotic cells when the mutated huntingtin was expressed alone (Fig. 4*A*). These data further support a role for MLK2 in the mediation of neuronal toxicity induced by polyglutamine-expanded huntingtin.

Our data show that the N-terminal proline-rich region of huntingtin interacts with MLK2, and the polyglutamine expansion interferes with this interaction. These results suggest that the polyglutamine expansion may lead to an increase in free MLK2 proteins, which are constitutively active and cell toxic (14). If this hypothesis is true, over-expression of the N terminus of normal huntingtin, which binds to free MLK2 proteins, should be able to overcome the neuronal toxicity induced by MLK2 and polyglutamine-expanded huntingtin. We prepared a construct encoding a small region of the normal huntingtin N terminus, containing a 16 polyglutamine repeat and the proline-rich region. As shown in Fig. 4*B*, co-expression of this N-terminal fragment of normal huntingtin significantly attenuated neuronal toxicity mediated by MLK2 and by the mutated huntingtin with 48 polyglutamine repeats. Over 50% of neurons remained viable at 48 h post-transfection, compared with less than 20% of viable cells when the N-terminal fragment was not co-expressed (Fig. 4*B*). These studies support our hypothesis that the polyglutamine expansion in huntingtin may interfere with its interaction with MLK2 thereby leading to an increase of free MLK2 proteins that in turn mediate JNK activation and neuronal apoptosis.

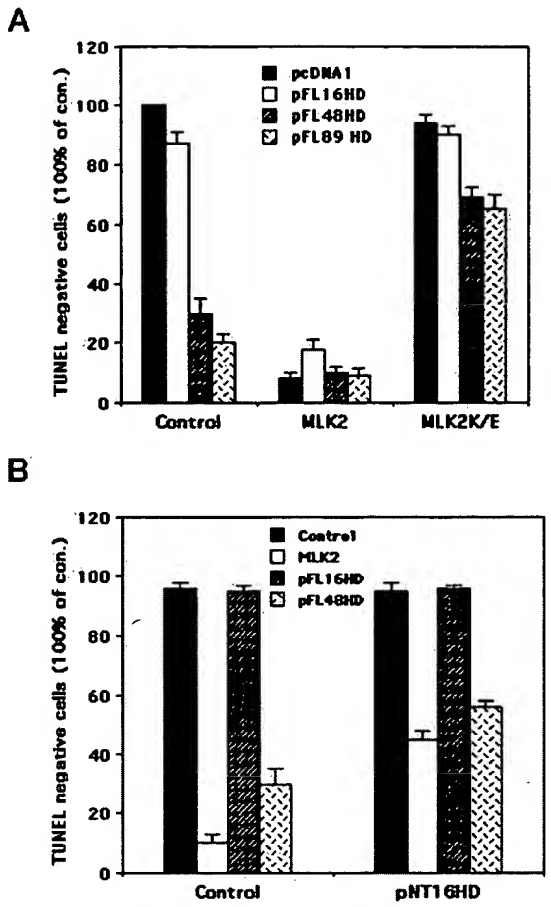

FIG. 4. **MLK2 may be partially involved in polyglutamine-expanded huntingtin-mediated neuronal apoptosis.** HN33 cells were transiently transfected with different plasmids as indicated in the figure. Cells were fixed at 48 h post-transfection, and TUNEL staining was carried out. TUNEL-negative cells were counted, and the number of TUNEL-negative cells transfected with vector (pcDNA1 + pRK5) was designated as 100%. Data are the average of three independent experiments. *A*, co-expression of dominant negative MLK2 significantly attenuated neuronal toxicity mediated by polyglutamine-expanded huntingtin. Full-length huntingtin constructs containing 16, 48, or 89 CAG repeats (1) were transfected alone or co-transfected with wild-type or dominant negative MLK2 (*MLK2K/E*) followed by TUNEL staining. *B*, over-expression of the N terminus of normal huntingtin overcame neuronal toxicity induced by MLK2 and by polyglutamine-expanded huntingtin. The expression vector for the N terminus of normal huntingtin with 16 CAG repeats (*pNT16HD*) was co-transfected with MLK2 or full-length huntingtin with 48 CAG repeats as indicated in the figure. TUNEL-negative cells were counted as described above.

DISCUSSION

In the present study, we demonstrate that MLK2, an upstream activator of the JNK pathway, is involved in JNK activation and neuronal apoptosis mediated by polyglutamine-expanded huntingtin (1). The polyglutamine expansion decreases the association of huntingtin with MLK2 leading to an increase in unregulated MLK2 proteins that, being constitutively active, cause JNK activation and neuronal toxicity. Co-expression of dominant negative MKK7 and SEK1, the downstream effectors of MLK2, blocks JNK activation and neuronal apoptosis induced by MLK2 and by polyglutamine-expanded huntingtin. Additionally, co-expression of dominant negative MLK2 significantly attenuated JNK activation and neuronal toxicity mediated by the polyglutamine expanded huntingtin. Finally, over-expression of a normal huntingtin N-terminal protein significantly attenuated neuronal toxicity induced by both MLK2 and polyglutamine-expanded huntingtin. These results show that MLK2-mediated cellular signaling cascades may play a significant role in neuronal death induced by polyglutamine-expanded huntingtin in HN33 cells.

Because huntingtin is a ubiquitously expressed protein, whereas the pathology of HD is restricted to the brain, it is likely that huntingtin binds to proteins that are largely found in the brain, and the polyglutamine expansion alters huntingtin's ability to interact with these proteins thereby resulting in activation of neurotoxic pathways. MLK2 being almost exclu-

sively expressed in the brain could provide a partial explanation for why the polyglutamine-expanded huntingtin is selectively toxic to neurons. MLK2 is a strong activator of the JNK pathway that is now known to couple a variety of cell-toxic stimuli, leading to neuronal apoptosis (21, 22). Because MLK2 is a constitutively active kinase, and free MLK2 is the active form (14), any alteration of the amount of bound MLK2 is likely to lead to activation of MLK2-mediated signaling cascades and neuronal toxicity. We show that normal huntingtin proteins from normal or HD human cortex tissues specifically bind to wild-type but not the mutated SH3 domain of MLK2, and under the same conditions, the mutated huntingtin proteins from HD patient cortex tissues only weakly bound to the SH3 domain of MLK2. Moreover, over-expression of the N terminus of normal huntingtin, which binds and decreases free MLK2 proteins in HN33 cells, can significantly inhibit neuronal toxicity induced by MLK2 and by the mutated huntingtin, further supporting the notion that huntingtin's N terminus interacts with the SH3 domain of MLK2, and the ability to bind to the kinase may be impaired upon polyglutamine expansion in huntingtin.

The polyglutamine expansion apparently alters the physical properties of huntingtin. The mobility of the mutated huntingtin on SDS-polyacrylamide gel electrophoresis is clearly decreased (5), and huntingtin's N terminus protein carrying an expanded polyglutamine stretch forms amyloid-like protein aggregates both *in vitro* and *in vivo* (23, 24). Because the N-terminal proline-rich region is adjacent to the polyglutamine stretch, it is possible that the polyglutamine expansion may alter the binding properties of this proline-rich region. Our group previously found that normal huntingtin is associated with epidermal growth factor receptor signaling complexes through binding to the SH3 domains of Grb2 and RasGAP, and this association is regulated by activation of the epidermal growth factor receptor (10). Recently, other groups have also reported that huntingtin binds to Grb2-like SH3 domain-containing proteins, and the N-terminal proline-rich region mediates these interactions (10–11). Our results from the current study are consistent with these reports (10–11). In addition, we show that the ability of this proline-rich region to bind to the SH3 domain of MLK2 is impaired upon expansion of the polyglutamine stretch. The interaction of proline-rich motifs with SH3 domains is not a highly selective event (9). Thus, if the polyglutamine expansion in huntingtin interferes with its interaction with the SH3 domain of MLK2, it may inhibit its association with other SH3 domain-containing proteins. Perhaps the normal function of huntingtin is the modulation of the cellular signaling network by sequestering these SH3 domain-containing signaling proteins. When the ability of huntingtin to interact with SH3 domains is impaired, polyglutamine-expanded huntingtin may be disassociated from the microtubule complex where most SH3 domain-containing proteins are found, leading to the re-arrangement of SH3 domain-containing protein-associated signaling complexes, which may subsequently result in an imbalance of cellular signaling networks and neuronal death. Incidentally, huntingtin's N terminus with an expanded length of polyglutamine repeat forms nuclear inclusions in the brains of HD patients or in cultured cells (25), indicating that some mutated huntingtin proteins are translocated and no longer co-present with MLK2 or other SH3 domain-containing proteins in the cytoplasm.

It is clear now that striatal medium-spiny neurons, which die first in HD, lack endogenous huntingtin. Thus, diffusible neurotoxic factors may play an important role in early neuronal loss in HD. Our previous studies and current results are consistent with this notion; expression of the mutated huntingtin or MLK2 induces not only apoptotic cell death of transfected HN33 cells but also non-transfected cells. Because JNK activation has been reported to mediate free radical production (26), it is possible that polyglutamine-expanded huntingtin may mediate free radical production via activation of the MLK2-JNK pathway. In summary, our current studies show that activation of MLK2-mediated signaling cascades may be partially responsible for neuronal loss in HD, and an inhibitor of MLK2 may be useful for the prevention of neuronal loss in HD.

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REFERENCES

1. Liu, Y. F. (1998) *J. Biol. Chem.* **273,** 28873–28877
2. Huntington's Disease Collaborative Research Group (1993) *Cell* **72,** 971–983
3. Martin, J. B., and Gusella, J. F. (1986) *N. Engl. J. Med.* **315,** 1267–1276
4. Ferrante, R. J., Kowall, N. W., Beal, M. F., Richardson, E. P., Jr., Bird, E. D., and Martin, J. B. (1985) *Science* **230,** 561–563
5. Trottier, Y., Devys, D., Imbert, G., Saudou, F., An, I., Lutz, Y., Weber, C., Agid, Y., Hirsch, E. C., and Mandel, J. L. (1995) *Nat. Genet.* **10,** 104–110
6. Fusco, F. R., Chen, Q., Lamoreaux, W., Figueredo-Cardenas, G., Jiao, Y., Coffiman, J. A., Surmerier, D. J., Honig, M. G., Carlock, L. R., and Reiner, A. (1999) *J. Neurosci.* **19,** 1189–1202
7. Gutekunst, C.-A., Levey, A. J., Hellman, C. J., Whsley, W. L., Yi, H., Nash, R., Rees, H. D., Madden, J. J., and Hersch, S. M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 8710–8714
8. Stine, O. C., Pleasant, N., Franz, M. L., Abbott, M. H., Folstein, S. E., and Ross, C. A. (1993) *Hum. Mol. Genet.* **2,** 1547–1549
9. Sudol, M. (1998) *Oncogene* **17,** 1469–1474
10. Liu, Y. F., Deth, R. C., and Devys, D. (1997) *J. Biol. Chem.* **272,** 8121–8124
11. Sitter, A., Wälter, S., Wedemeyer, N., Hasenbank, R., Scherzinger, E., Eickhoff, H., Bates, G. P., Lehrach, H., and Wanker, E. E.(1998) *Mol. Cell* **2,** 427–436
12. Faber, P. W., Barnes, G. T., Srinidhi, J., Chen, J., Gusella, J. F., and MacDonald, M. E. (1998) *Hum. Mol. Genet.* **7,** 1463–1474
13. Dorow, D. S., Devereux, L., Tu, G.-F., Price, G., Nicholl, J. K., Sutherland, G. R., and Simpson, R. J. (1995) *Eur. J. Biochem.* **234,** 492–500
14. Nagata, K. Puls, A., Futter, C., Aspenstrom, P., Schaefer, E., Nakata, T., Hirokawa, N., and Hall, A. (1998) *EMBO J.* **17,** 149–158
15. Hirai, S., Katoh, M., Terada, M., Kyriakis, J. M., Zon, L. I., Rana, A., Avruch, J., and Ohno, S. (1997) *J. Biol. Chem.* **272,** 15167–15173
16. Hirai, S., Noda, K., Moriguchi, T., Nishida, E, Yamashita, A., Deyama, T., Fukuyama, K., and Ohno, S. (1998) *J. Biol. Chem.* **273,** 7406–7412
17. Gupta, S., Campbell, D., Derijard, B., and Davis, R. J (1995) *Science* **267,** 389–393
18. Sanchez, I., Hughes, R. T., Mayer, B. J., Yee, K., Woodgett, J. R., Avruch, J., Kyriakis, J. M., and Zon, L. I. (1994) *Nature* **380,** 75–79
19. Tournier, C., Whitmarsh, A. J., Cavanagh, J., Barrett, T., and Davis, R. J. (1997) *Proc. Natl. Acad. Sci.* U. S. A. **84,** 7337–7342
20. Phelan, D. R., Loveland, K. L., Devereux, L., and Dorow, D. S. (1999) *Mol. Reprod. Dev.* **52,** 135–140
21. Schwarzschild, M. A., Cole, R. L., and Hyman, S. E. (1997) *J. Neurosci.* **17,** 3455–3466
22. Eilers, A., Whitfield, J., Banij, C., Rubin, L. L., and Ham, J. (1998) *J. Neurosci.* **18,** 1713–1724
23. 29 DiFiglia, M., Sapp, E., Chase, K. O., Davies, S. P., Bates, G. P., Vonsattel, J. P., and Aronin, N. (1997) *Science* **277,** 1990–1993
24. Scherzinger, E., Lurz, R., Turmaine, M., Mangiarini, L., Hollenbach, B., Hasenbank, R., Bates, G. P., Davies, S. W., Lehrach, H., and Wanker, E. E. (1997) *Cell* **90,** 549–559
25. Saudou, F., Finkbeiner, S., Deys, D., Greenberg, M. E. (1998) *Cell* **95,** 55–66
26. Go, Y. M., Patel, R. P., Maland, M. C., Park, H., Beckman, J. S., Darley-Usmar, V. M., Jo, H. (1999) *Am. J. Physiol.* **277,** H1647–H1653